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Mini-Review

Novel σ^B regulation modules of Gram-positive bacteria involve the use of complex hybrid histidine kinases

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A common bacterial strategy to cope with stressful conditions is the activation of alternative sigma factors that control specific regulons enabling targeted responses. In the human pathogen *Bacillus cereus*, activation of the major stress-responsive sigma factor σ^B is controlled by a signalling route that involves the multi-sensor hybrid histidine kinase RsbK. RsbK-type kinases are not restricted to the *B. cereus* group, but occur in a wide variety of other bacterial species, including members of the the low-GC Gram-positive genera *Geobacillus* and *Paenibacillus* as well as the high-GC actinobacteria. Genome context and protein sequence analyses of 118 RsbK homologues revealed extreme variability in N-terminal sensory as well as C-terminal regulatory domains and suggested that RsbK-type kinases are subject to complex fine-tuning systems, including sensitization and desensitization via methylation and demethylation within the helical domain preceding the H-box. The RsbK-mediated stress-responsive sigma factor activation mechanism that has evolved in *B. cereus* and the other species differs markedly from the extensively studied and highly conserved RsbRST-mediated σ^B activation route found in *Bacillus subtilis* and other low-GC Gram-positive bacteria. Implications for future research on sigma factor control mechanisms are presented and current knowledge gaps are briefly discussed.

Introduction

Bacteria use dedicated sets of sensory modules to tightly coordinate gene expression in response to environmental fluctuations. A commonly used sensory module is the two-component signal transduction system (TCS), which includes a transmembrane sensor histidine kinase (HK) and its cognate response regulator (RR). The mode of signal transduction by TCSs involves a phospho-transfer reaction between a conserved histidine and aspartate residue located in the HK phosphotransferase and RR receiver (REC) domain, respectively. RRs generally function as transcription factors that, upon phosphorylation, bind to specific sites on the DNA to alter the expression of the genes involved in adaptive responses (Hoch, 2000).

Another bacterial strategy for tight control of gene expression is the use of alternative sigma factors. In exponentially growing cells, most of the transcription is mediated by a 'housekeeping' sigma factor that is equivalent to σ^{70} in *Escherichia coli* and σ^A in *Bacillus subtilis*. However, under specific conditions, such as severe environmental stress, the housekeeping sigma factor gets replaced from the RNA polymerase by alternative sigma factors that recognize specific promoters and control specialized regulons (Gruber & Gross, 2003). One of the best-studied alternative sigma factors is the key stress-responsive sigma factor σ^B of low-GC Gram-positive bacteria of the genera *Bacillus*, *Listeria* and *Staphylococcus* (Hecker *et al.*, 2007; Price, 2002). Besides mediating the

Abbreviations: HK, histidine kinase; MCP, methyl-accepting chemotaxis protein; RR, response regulator; REC, RR receiver; TCS, two-component signal transduction system.

Six supplementary figures are available with the online version of this paper.

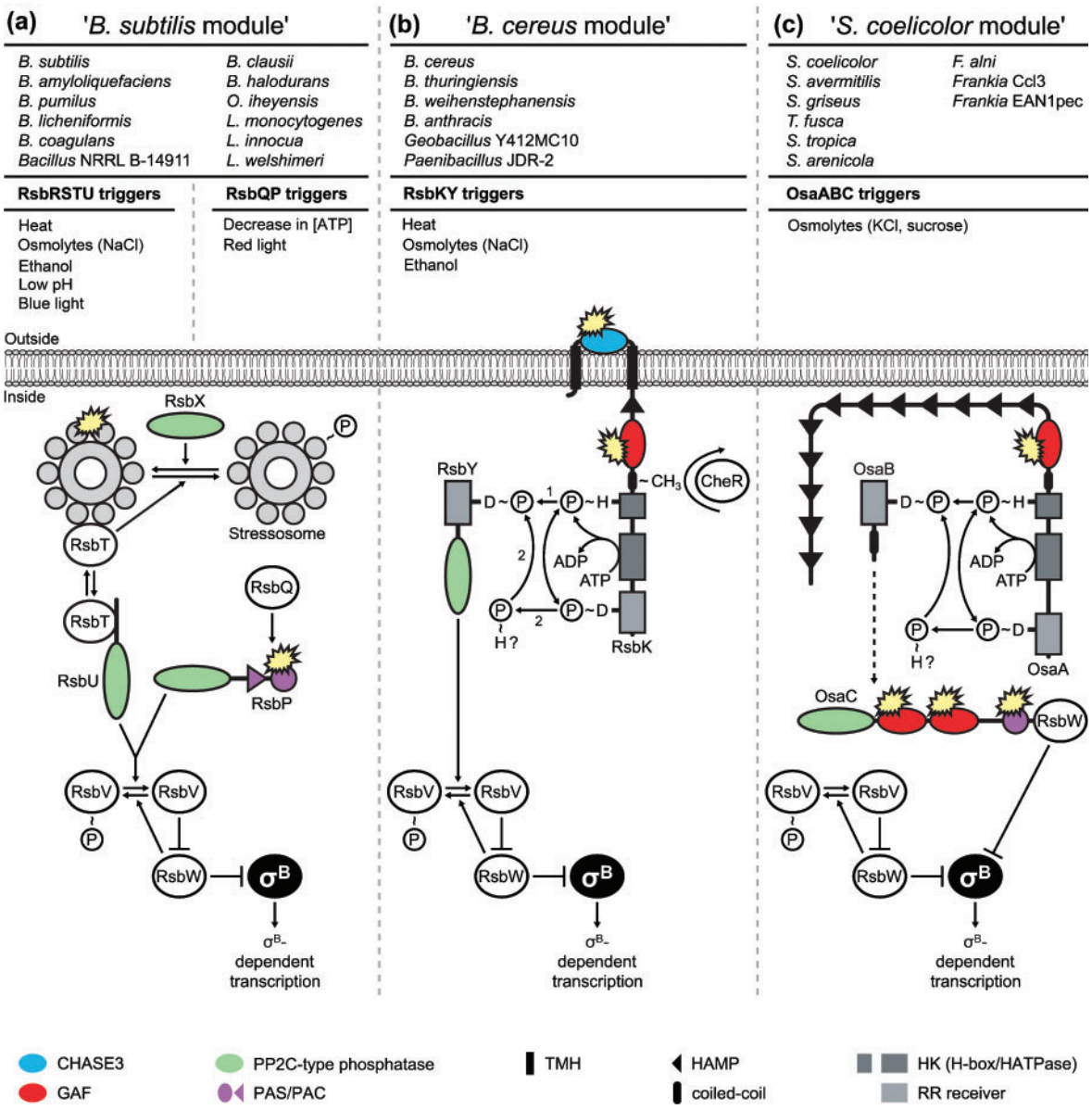

Fig. 1. Established and predicted signalling routes for the control of σ^B activity in different Gram-positive bacteria. Locations of potential stimulus perception are indicated by 'explosion' symbols. (a) The extensively studied '*B. subtilis* module' for the control of σ^B. The *B. subtilis* stressosome is an ~1.8 MDa supramolecular complex that consists of multiple copies of RsbR and RsbS (Chen *et al.*, 2003; Delumeau *et al.*, 2006; Dufour *et al.*, 1996; Marles-Wright *et al.*, 2008). Different environmental stresses and signals are thought to stimulate RsbT kinase activity towards its stressosome substrates (Akbar *et al.*, 2001; Ávila-Pérez *et al.*, 2006; Gaidenko *et al.*, 2006; Kim *et al.*, 2004; Voelker *et al.*, 1995). This leads to dissociation of RsbT from the stressosome to activate RsbU and subsequently σ^B (Delumeau *et al.*, 2004). Return of the stressosome to the unphosphorylated state is achieved by RsbX (Chen *et al.*, 2004). A second *B. subtilis* σ^B activation pathway involves the hydrolase or acyltransferase RsbQ and the PP2C-type phosphatase RsbP, which are required for triggering σ^B activity in red light and conditions of energy stress (Ávila-Pérez *et al.*, 2010; Brody *et al.*, 2001, 2009; Vijay *et al.*, 2000). Note that RsbRST-dependent σ^B activation is conserved across several other low-GC Gram-positive bacteria, but that RsbQP-dependent σ^B activation has so far only been found in *B. subtilis*. (b) Model for the control of σ^B in *B. cereus*. Upon different stresses, RsbK most likely 'auto'-phosphorylates a conserved histidine residue within its H-box. The phosphoryl group is then either transferred directly to a conserved asparate within the REC domain of RsbY (route 1), or shuttled to RsbY indirectly (route 2) via RsbK's own REC domain and a potential phosphotransferase protein (marked 'H?') (de Been *et al.*, 2010). In the former scenario, the RsbK REC may play a role in fine-tuning RsbK kinase activity. Phosphoryl transfer within RsbK is therefore shown by a double arrow. Phosphorylation of RsbY most likely activates its PP2C domain, which dephosphorylates RsbV, ultimately resulting in the activation of σ^B (van Schaik *et al.*, 2005). An additional fine-tuning system may include CheR-mediated methylation processes of RsbK (this study). Other *B.*

cereus group members and bacteria such as *Geobacillus* and *Paenibacillus* spp. also appear to use the RsbKY module for the control of σ^B. (c) Model for the control of σ^B in *Streptomyces coelicolor*, which involves the RsbK homologue OsaA. In post-osmotic shock conditions, OsaA most likely activates its putative partner RR OsaB, analogous to how RsbK may activate RsbY (b). In turn, OsaB may activate OsaC either directly or indirectly (dashed line). The RsbW-like anti-sigma factor domain of OsaC is required for preventing continued activation of σ^B (Fernández Martínez *et al.*, 2009). OsaABC modules occur in several actinobacteria, where they may also control σ^B activity (this study).

general stress response, σ^B plays an important role in virulence in the human pathogens *Listeria monocytogenes* and *Staphylococcus aureus* (Chaturongakul *et al.*, 2008; Novick, 2003), and to a lesser extent in *Bacillus anthracis* (Fouet *et al.*, 2000). Sigma factors equivalent to σ^B have also been found in high-GC Gram-positive bacteria, including *Mycobacterium tuberculosis* and *Streptomyces* species (Mittenhuber, 2002). In *Streptomyces coelicolor*, σ^B acts in a complex network of several paralogous sigma factors, where it plays a role in osmotic and oxidative stress responses, as well as cellular differentiation and the production of antibiotics (Cho *et al.*, 2001; Lee *et al.*, 2005; Viollier *et al.*, 2003).

The σ^B network has been studied best in the model low-GC Gram-positive *B. subtilis*. In *B. subtilis*, σ^B activity is controlled by RsbVW partner-switching, a mechanism that is highly conserved in species that contain σ^B (Fig. 1, bottom). Under non-stress conditions, σ^B is held in an inactive state complex by the anti-sigma factor RsbW. Release of σ^B from RsbW is accomplished by the anti-anti sigma factor RsbV, which, upon dephosphorylation, sequesters RsbW. In addition, RsbW acts as a kinase of RsbV, thereby providing a negative feedback on σ^B activation. Under stress conditions, RsbV is dephosphorylated by one or more specific PP2C-type phosphatases, resulting in the sequestration of RsbW and the activation of σ^B (Hecker *et al.*, 2007; Price, 2002).

Whereas RsbVW-mediated control of σ^B is highly conserved, the N-terminal input domains of the PP2C-type phosphatases vary considerably across species (van Schaik & Abee, 2005). For example, *B. subtilis* contains two σ^B-activating PP2C-type phosphatases, RsbP and RsbU. Energy stress is probably signalled through RsbP, which contains an N-terminal PAS sensory domain (Brody *et al.*, 2001, 2009; Vijay *et al.*, 2000), while environmental stress (i.e. heat, osmolytes, ethanol, low pH) is signalled through RsbU, which contains an N-terminus that interacts with the regulator RsbT, which in turn interacts with the RsbR- and RsbS-containing supramolecular 'stressosome' (Akbar *et al.*, 2001; Chen *et al.*, 2003; Delumeau *et al.*, 2006; Dufour *et al.*, 1996; Kim *et al.*, 2004; Marles-Wright *et al.*, 2008) (Fig. 1a).

In the human pathogen *Bacillus cereus*, the mechanism of σ^B activation has only been studied more recently (see Fig. 1b for the current model). It has been shown that σ^B activation is governed by a single PP2C-type phosphatase, RsbY, which carries an N-terminal REC domain (van Schaik *et al.*, 2005). This suggested the existence of a partner HK acting on RsbY. Indeed, we have recently identified the hybrid kinase RsbK (BC1008) as a potential partner in the σ^B-mediated stress response of *B. cereus* (de Been *et al.*, 2010). RsbK contains both HK and RR domains, and the *rsbK* gene is located close to *sigB* on the genome. A genome survey indicated that RsbK and RsbY should constitute one functional module for the control of σ^B activity in members of the *B. cereus* group, including the pathogens *Bacillus thuringiensis* and *B. anthracis* and the psychrotolerant *B. weihenstephanensis*. Orthologous RsbKY signalling modules were found in four other bacilli outside the *B. cereus* group. However, the RsbKY modules of these other bacilli were not genomically associated with *sigB* (de Been *et al.*, 2010). To explore the occurrence of RsbK- and RsbY-like proteins outside the *B. cereus* group, we searched available microbial and eukaryotic genome sequences for the presence of RsbK- and RsbY-type signalling domains. Subsequent phylogenetic and gene context analyses revealed that signalling modules involving RsbK (and sometimes also RsbY) homologues are present in several other low-GC as well as high-GC Gram-positive bacteria and could potentially regulate σ^B-like sigma factors. Based on these results, we propose that, besides the well-characterized and conserved σ^B activation pathway of *B. subtilis*, the use of RsbK-type hybrid kinases is another common bacterial strategy to regulate stress-responsive σ^B(-like) sigma factors.

RsbK-type hybrid kinases occur in a wide variety of bacterial species

To map the occurrence of RsbK and RsbY homologues in other species, a similar approach was followed as described previously (de Been *et al.*, 2006, 2010). This approach included a straightforward BLAST search with the HK phosphotransferase (RsbK) and REC (RsbK and RsbY) domain sequences. As these types of domains are characteristic for all TCSs and are easily recognized, such a search should yield all potential candidates. In fact, it has been shown that these domains contain enough information density to enable their use in classification and evolutionary studies (Alm *et al.*, 2006; Fabret *et al.*, 1999; Grebe & Stock, 1999). For each of the three domains, their protein BLAST hits were aligned and a bootstrapped neighbour-joining tree was built (see Supplementary Figs S1–S3, available with the online version of this paper). For assigning potential RsbK and RsbY homologues, we used a minimal bootstrap support of 30 %. In the case of RsbY, the procedure resulted in the identification of only six putative RsbY homologues outside the *B. cereus* group

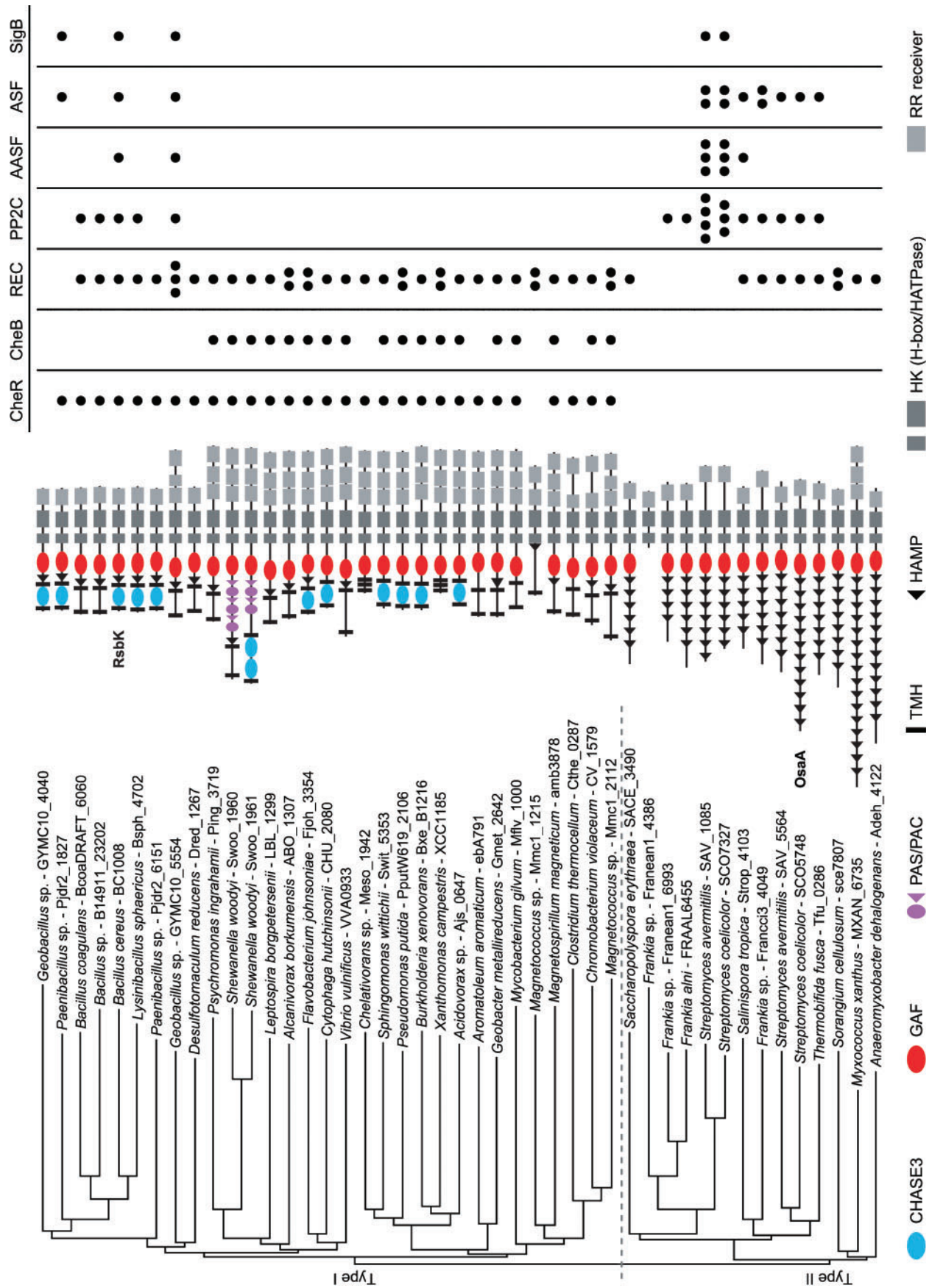
CheR
CheB
REC
PP2C
AASF
ASF
SigB
Geobacillus sp. - GYMC10_4040
Paenibacillus sp. - Pjdr2_1827
Bacillus coagulans - BcoaDRAFT_6060
Bacillus sp. - B14911_23202
Bacillus cereus - BC1008
Lysinibacillus sphaericus - Bsph_4702
Paenibacillus sp. - Pjdr2_6151
Geobacillus sp. - GYMC10_5554
Desulfotomaculum reducens - Dred_1267
Psychromonas ingrahamii - Ping_3719
Shewanella woodyi - Swoo_1960
Shewanella woodyi - Swoo_1961
Leptospira borgpetersenii - LBL_1299
Alcanivorax borkumensis - ABO_1307
Flavobacterium johnsoniae - Fjoh_3354
Cytophaga hutchinsonii - CHU_2080
Vibrio vulnificus - VVA0933
Chelativorans sp. - Meso_1942
Sphingomonas wittichii - Swit_5353
Pseudomonas putida - PputW619_2106
Burkholderia xenovorans - Bxe_B1216
Xanthomonas campestris - XCC1185
Acidovorax sp. - Ajs_0647
Aromatoleum aromaticum - ebA791
Geobacter metallireducens - Gmet_2642
Mycobacterium gilvum - Mflv_1000
Magnetococcus sp. - Mmc1_1215
Magnetospirillum magneticum - amb3878
Clostridium thermocellum - Cthe_0287
Chromobacterium violaceum - CV_1579
Magnetococcus sp. - Mmc1_2112
Saccharopolyspora erythraea - SACE_3490
Frankia sp. - Franean1_4386
Frankia sp. - Franean1_6993
Frankia alni - FRAAL6455
Streptomyces avermitilis - SAV_1085
Streptomyces coelicolor - SCO7327
Salinispora tropica - Strop_4103
Frankia sp. - Francci3_4049
Streptomyces avermitilis - SAV_5564
Streptomyces coelicolor - SCO5748
Thermobifida fusca - Tfu_0286
Sorangium cellulosum - sce7807
Myxococcus xanthus - MXAN_6735
Anaeromyxobacter dehalogenans - Adeh_4122
RsbK
OsaA
Type I
Type II
CHASE3
GAF
PAS/PAC
TMH
HAMP
HK (H-box/HATPase)
RR receiver

Fig. 2. Relationship between RsbK homologues in terms of their HK and RR domains, overall domain architecture and associated genomic context. Left: reduced version of the bootstrapped neighbour-joining tree that was built using the HK phosphotransferase and REC domain sequences (concatenated) of 118 RsbK-type HKs (Supplementary Fig S4). Species and protein names (separated by '-') are indicated. The tree represents RsbK and its 89 closest homologues, including OsaA of *S. coelicolor* (Bishop *et al.*, 2004). Because the tree was rendered non-redundant, only 45 proteins are displayed (e.g. *B. cereus* RsbK represents eight additional proteins, all from other *B. cereus* group members). This tree was rooted on Bphy_5629 of *Burkholderia phymatum*, which clustered with the remaining 28 more distantly related RsbK homologues (Supplementary Fig. S4), including MXAN_0712 of *Myxococcus xanthus* (Shi *et al.*, 2008). The dashed line separates type I (above the line) and type II (below the line) RsbK homologues. Middle: domain architecture of RsbK homologues as defined by SMART (Letunic *et al.*, 2009). Right: genomic associations of *rsbK* homologues with genes encoding CheR, CheB, RR (REC), PP2C-type phosphatase, anti-anti sigma factor (AASF), anti-sigma factor (ASF) and σ^B (SigB)-like proteins. The number of dots indicates the number of genomic associations. In the case of CheR-, CheB- and RR-encoding genes, only the operons (max. intergenic region 200 bp) containing the *rsbK* homologues were considered. Exceptions were made for *rsbK* and its orthologues in bacilli and for *osaA* and its orthologues in actinobacteria, where the 'known' partner RRs (RsbY and OsaB, respectively) are encoded in operons that flank the *rsbK*/*osaA* operons. In the case of sigma factor- and sigma factor regulator-encoding genes, the gene neighbourhoods (±5 kb) of the *rsbK* homologues were considered. Note that the columns displaying the number of AASFs and ASFs also include RsbR/S- and RsbT-like proteins, respectively. ASFs were identified by the presence of a HATPase_c domain. Because this domain also occurs in other protein types, ASFs were only assigned as such when they were genomically associated with sigma factors, PP2C-type phosphatases or AASFs.

(Supplementary Fig. S3). However, in the case of RsbK, we identified a set of 118 putative RsbK homologues across 101 bacterial species, including species from the phyla Proteobacteria (71 RsbK homologues), Firmicutes (19) Actinobacteria (17), Cyanobacteria (7), Bacteroidetes (2) and undefined (2). No RsbK homologues were found in Archaea and Eukaryotes. As the neighbour-joining trees constructed from both RsbK domains were almost identical (Supplementary Figs S1 and S2), we built a concatenated tree. A reduced version, representing the closest homologues of *B. cereus* RsbK, is shown in Fig. 2 (the complete concatenated tree is shown in Supplementary Fig. S4). The hybrid HKs of *Bacillus coagulans* 36D1, *Lysinibacillus sphaericus* C3-41, *Bacillus* B14905 and *Bacillus* NRRL B-14911 appeared most similar (bootstrap 95.8 %) to RsbK of *B. cereus*. The other RsbK homologues found in the low-GC Gram-positives were in *Paenibacillus* JDR-2 (2×), *Geobacillus* Y412MC10 (2×), *Desulfotomaculum reducens* MI-1 and *Clostridium thermocellum* ATCC 27405, although the last one seems more distantly related.

RsbK-type HKs display extremely variable sensory and C-terminal regions

Sequence analysis of the RsbK homologues revealed the presence of several N-terminal HK sensory domains (Fig. 2), including CHASE3, GAF and PAS/PAC domains, of which the latter two have been implicated in small ligand/cyclic nucleotide binding and redox/light/metabolite sensing, respectively (Galperin, 2004). The GAF sensory domain was highly conserved in the RsbK homologues and was always found next to, and N-terminally from, the HK phosphotransferase domain. In addition, almost all RsbK homologues contained one to several putative HAMP domains, which are thought to link N-terminal sensory domains with intracellular phosphotransferases (Hulko *et al.*, 2006).

Based on the N-terminal sensory regions, a subdivision could be made into two RsbK-types: type I, containing transmembrane helices and putative membrane-associated extracellular sensory domains (this type includes *B. cereus* RsbK); and type II, lacking transmembrane helices and containing a multitude of HAMP linker regions (Fig. 2). Even within the two types, high variability was observed between the different N-terminal regions. For example, in *Shewanella woodyi* two type I RsbK-like HKs were identified (Swoo1960 and Swoo1961), which were highly related in terms of their phosphotransferase and GAF domains, but which displayed marked differences with respect to their N-terminal sensory domains. In type II RsbK homologues, the N-terminal regions showed extreme variability with respect to the number of detected HAMP domains per HK, which ranged from 4 to as many as 14. These findings are in agreement with a previous study in which it was shown that evolutionarily related HKs can contain very different N-terminal regions due to shufflings and duplications of sensory domains (Alm *et al.*, 2006).

A more surprising variability was observed in the number of predicted C-terminal REC domains, which ranged from one in most of the RbsK homologues found in low- and high-GC Gram-positive bacteria to as many as three in some of the other species. When considering the homologues that contained two or three REC domains, the C-terminal REC domain always appeared to be most similar to RsbK REC (~50 % identical), while the other REC domains were relatively dissimilar from RsbK REC (~25 % identical), the only exception being Mmc1_1215 of *Magnetococcus* MC-1.

Connecting the RsbK homologues to cognate RRs

In addition to RsbK of *B. cereus*, two of its homologues have been experimentally characterized. These include the type II homologues SCO5748 (OsaA) of *Streptomyces coelicolor* and MXAN_0712 of *Myxococcus xanthus*. OsaA of *S. coelicolor* and its putative cognate RR OsaB have been implicated to function in osmoadaptation, aerial mycelium formation and the coordination of antibiotic production (Bishop *et al.*, 2004), while MXAN_0712 of *M. xanthus* was shown to be essential in fruiting body formation and sporulation (Shi *et al.*, 2008). To obtain additional information about the potential biological role of the other RsbK homologues, especially with respect to the possible regulation of alternative sigma factors, we analysed the genomic regions surrounding RsbK-encoding genes. Genetic context, especially when found conserved across species, is a strong indicator of the biological role of a gene (Dandekar *et al.*, 1998; Overbeek *et al.*, 1999). Almost all RsbK homologues (~88 %) were genomically connected to one or more genes encoding an RR (Fig. 2). The domain composition of these RRs appeared highly variable, ranging from the 'classical' composition, containing an N-terminal REC and a C-terminal DNA-binding domain, to 'atypical' composition, containing a single REC domain or putative C-terminal guanylate cyclase-, cyclic di-GMP phosphodiesterase- and kinase-type output domains in addition.

Connections of RsbK homologues with CheR and CheB homologues

Interestingly, many RsbK homologues appeared to be genomically associated with genes encoding putative CheR (~60 % of the HKs) and CheB homologues (~42 %) (Fig. 2 and Supplementary Fig. S5). CheR and CheB have been extensively studied in *E. coli* and *B. subtilis*, where they play a role in the adaptation (i.e. sensitization and desensitization) of the chemotaxis machinery to persisting stimuli. CheR is a methyltransferase that methylates specific glutamate residues within methyl-accepting chemotaxis proteins (MCPs), while the methylesterase/deamidase CheB removes methyl groups from these proteins. MCPs function as stimulus receptors that transduce their signals to the chemotaxis regulator CheA. The methylation state of the MCPs influences this signalling activity and consequently influences flagellar rotation (Hazelbauer & Lai, 2010). The observed genomic association between RsbK-type HKs and the CheR/B homologues could imply a role for these HKs in bacterial chemotaxis. However, no evidence for such a role has been found so far. Therefore it is much more likely that the hybrid kinases themselves are the main target of these CheR/B homologues. In MCPs, methylation sites generally appear as glutamate (E) or glutamine (Q) pairs that are located in tandemly repeated heptads within coiled-coil regions. In the case of glutamine, the side chain is deamidated by CheB prior to its participation in the methylation cycle (Hazelbauer & Lai, 2010). Interestingly, sequence analysis of the RsbK homologues indeed revealed the presence of conserved glutamate and glutamine pairs. These conserved pairs were always found between the cytoplasmic GAF domain and the H-box and always occurred in tandemly repeated heptads (Supplementary Fig. S6). In fact, some of these heptads have recently been predicted to constitute a conserved helical domain (Anantharaman *et al.*, 2006). In a previous comparative study of bacterial MCPs (Le Moual & Koshland, 1996), predicted and confirmed methylation sites were assigned to positions 'b' and 'c' of the 'a-b-c-d-e-f-g' heptad repeat, according to the scheme of McLachlan & Stewart (1975). Similarly, we could assign the detected glutamate and glutamine pairs of the RsbK homologues to these positions, which were followed almost invariably by a leucine (L) at position 'd' (Fig. 3 and Supplementary Fig. S6). As compared to established methylation heptads in MCPs from *E. coli*, *Salmonella enterica* and *B. subtilis* (Le Moual & Koshland, 1996; Zimmer *et al.*, 2000), the heptads found in the RsbK homologues appeared to be different at positions 'a-d-e-f-g'. However, a recent study on *Thermotoga maritima* MCPs has revealed that CheR-mediated methylation heptads can indeed be distinct from the *E. coli*, *S. enterica* and *B. subtilis* consensus (Perez *et al.*, 2006) (Fig. 3).

On the basis of the above data, we conclude that the sensitivity of RsbK-type HKs to environmental signals probably can be modulated via CheR/B-homologue-mediated methylation/demethylation. This hypothesis is supported by the fact that the RsbK homologues that are genomically associated with the CheR/B homologues generally contain more putative methylatable pairs at the 'b-c' positions than those that are not associated with the CheR/B homologues (an average of 4.7 pairs per protein versus 2.2 pairs per protein, respectively; Supplementary Fig. S5).

RsbK homologues found in Gram-positive bacteria are connected to σ^B

The gene context analysis revealed that besides *rsbK* in the *B. cereus* group, several *rsbK* homologues found in low- and high-GC Gram-positive bacteria are located in gene clusters encoding one to several proteins related to the σ^B-mediated stress response. The clusters included genes

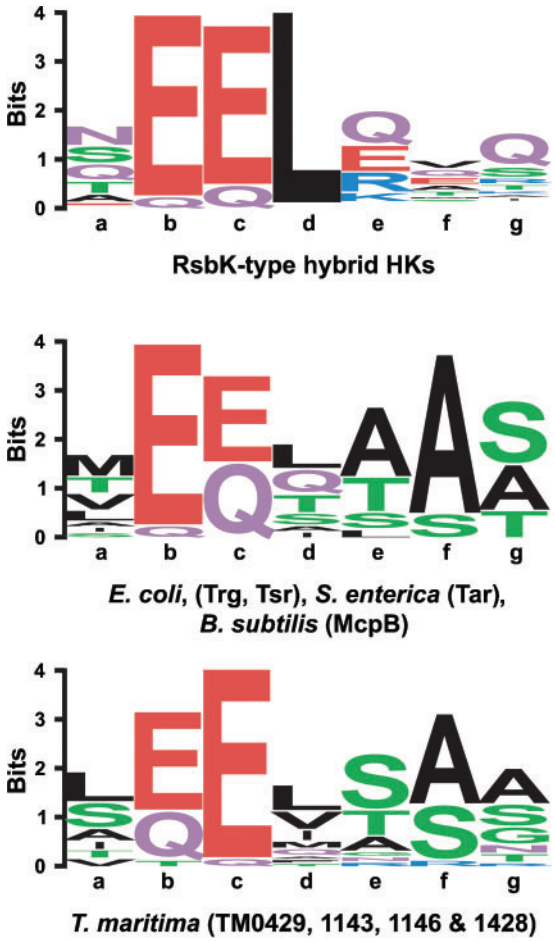

Fig. 3. Potential methylation sites in RsbK homologues. WebLogo (Crooks *et al.*, 2004) representations of the predicted RsbK-type HK methylation sites (top) and of confirmed methylation sites in MCPs of *Escherichia coli*, *Salmonella enterica*, *Bacillus subtilis* (middle) and *Thermotoga maritima* (bottom) are shown. Just like the methylatable residues (i.e. glutamate and glutamine pairs) of MCPs, the predicted methylatable residues of the RsbK homologues could be assigned to positions 'b' and 'c' of the 'a-b-c-d-e-f-g' heptad repeat, according to the scheme of McLachlan & Stewart (1975). The top image was constructed with the highlighted heptads starting from position 10 in each sequence shown in Supplementary Fig. S6. References for known methylation sites: *E. coli* Tsr, Trg and *S. enterica* Tar (reviewed by Le Moual & Koshland, 1996); *B. subtilis* McpB (Zimmer *et al.*, 2000); *T. maritima* MCPs (Perez *et al.*, 2006).

encoding PP2C-type phosphatases, RsbV, RsbW, RsbR, RsbS and RsbT. Moreover, four of these gene clusters (excluding *B. cereus*) also encode a putative σ^B-like sigma factor (Fig. 2). Some of the *rsbK* homologues, such as *fraal6455* in *Frankia alni* ACN14a, were found in gene clusters that encode a single putative σ^B-related regulator, while other *rsbK* homologues are located in gene clusters that encode multiple partner-switching proteins for the potential control of σ^B activity. For example, in *Streptomyces coelicolor* the *rsbK* homologue *sco7327* is located in a gene cluster that encodes as many as three potential PP2C-type phosphatases, an anti-sigma factor antagonist (RsbV), an RsbRST module and the alternative sigma factor σ^M, which is related to σ^B (Lee *et al.*, 2005). In addition, one of the PP2C phosphatases was found to be fused to an N-terminal ATPase and thus could function as an anti-sigma factor.

Another interesting gene cluster is present in *Geobacillus* Y412MC10, where the *rsbK* homologue *gymc10_5554* directly flanks an *rsbY*-like gene, similar to what is found in the *B. cereus* group. Other genes in its direct neighbourhood are *rsbV*, *rsbW* and *sigB*, but also *yflT* and *corA*. The latter two genes encode a putative general stress protein and an Mg^{2+}/Co^{2+} transporter, respectively. In fact, the orthologues of these genes in *B. cereus* (*bc0998* and *bc3129*, respectively) have been implicated previously in the σ^B-mediated stress response (de Been *et al.*, 2010). Finally, the RsbK homologue Pjdr2_1827 of *Paenibacillus* JDR-2 is likely to be involved in σ^B regulation because it is associated with *rsbW* and *sigB* and because the *Paenibacillus* genome harbours an operon encoding RsbY (Pjdr2_1863), RsbV, RsbW and YflT. This operon has probably 'jumped' to another location in the genome, as it is flanked by putative transposase- and integrase-encoding elements.

Novel activation routes for σ^B in Gram-positive bacteria

As described above, the RsbK-type HK OsaA and its putative cognate RR OsaB of *Streptomyces coelicolor* have been implicated in osmoadaptation, cellular differentiation and the production of antibiotics (Bishop *et al.*, 2004). In *S. coelicolor*, these processes are also controlled by a seemingly complex network of σ^B-like sigma factors (Cho *et al.*, 2001; Lee *et al.*, 2005; Viollier *et al.*, 2003). Indeed, experimental support for a functional link between OsaAB and a σ^B-like sigma factor (in this case σ^B itself) was recently provided (Fernández Martínez *et al.*, 2009). It was shown that *osaC*, which flanks *osaA* and *osaB* and is divergently transcribed from *osaA*, encodes a regulatory protein that contains an N-terminal RsbW-like domain, followed by PAS and GAF sensory domains and a PP2C-type phosphatase domain. The OsaC RsbW-like domain was demonstrated to interact with σ^B and to function as a σ^B anti-sigma factor. Furthermore, it was found that *osaB* is induced upon osmotic shock in a σ^B-dependent manner and that OsaC is essential for returning *osaB* and *sigB* expression levels back to 'normal' after osmotic shock (Fernández Martínez *et al.*, 2009).

Besides OsaC, another 'more classical' RsbW protein has been characterized in *S. coelicolor*. This protein (RsbA) acts in an RsbVW-like partner-switching module for the control of σ^B (Lee *et al.*, 2004). Whereas *rsbA* is located in the *sigB* operon, *rsbV* is located in a gene cluster encoding multiple putative sigma factor regulators as well as the σ^B paralogue, σ^M. Interestingly, this gene cluster also

includes *sco7327*, the second *rsbK* homologue of *S. coelicolor* next to *osaA* (Fig. 2). These findings indicate a complex regulatory connection between σ^B and σ^M, a connection that has been partly confirmed (Lee *et al.*, 2005). It is possible that SCO7327 controls σ^M activity, in conjunction with the genomically associated RsbRST module. However, the fact that it is genomically linked to *rsbV* may also suggest a role for this RsbK homologue in controlling σ^B activity.

Based on the above findings, it seems likely that *osaABC* encodes one functional module for the (post-osmotic shock) control of σ^B and perhaps other related sigma factors. Further support for OsaABC being one functional module comes from the fact that an *osaC* deletion mutant displayed a phenotype comparable to that of the *osaA* and *osaB* deletion mutants, at least with respect to osmoadaptation and cellular differentiation (Fernández Martínez *et al.*, 2009). One question that needs answering is how signals are transferred to OsaC. It has been suggested that OsaB may interact with other proteins via its C-terminal coiled-coil region (Fernández Martínez *et al.*, 2009). Considering this, we propose that after osmotic shock, the RsbK homologue OsaA triggers its partner RR OsaB to transduce its signals to OsaC via direct or indirect protein–protein interactions. In turn, this would activate the OsaC RsbW domain, thus preventing continued activation of σ^B (Fig. 1c). Another important question is what the C-terminal PP2C-type phosphatase domain of OsaC is doing. Since the *osaC* deletion mutant was not disturbed in its induction of σ^B upon osmotic shock, the PP2C-type phosphatase domain at least does not seem to dephosphorylate RsbV under those conditions (Fernández Martínez *et al.*, 2009).

The proposed model for σ^B regulation in *S. coelicolor* A3(2) also holds for the high-GC Gram-positive bacteria *Streptomyces avermitilis*, *Streptomyces griseus*, *Thermobifida fusca*, *Salinispora tropica*, *Salinispora arenicola*, and several *Frankia* species, which all contain the OsaABC module. As pointed out in this study, the above actinobacterial modules display strong similarities to the σ^B-regulating RsbKY module of the *B. cereus* group and possible other σ^B-regulating modules of Gram-positive bacteria. This strongly suggests that the use of RsbK/OsaA-type hybrid HKs is a common strategy for Gram-positive bacteria to control the activity of (σ^B-like) alternative sigma factors. As summarized in Fig. 1, this strategy is altogether different from the well-characterized σ^B activation pathway in *B. subtilis*.

Conclusions

Based on conserved gene context analysis, we suggest that RsbK(Y)-mediated regulation of alternative sigma factors is not restricted to members of the *B. cereus* group, but is used by several other low- as well as high-GC Gram-positive bacteria. In addition, we show that RsbK-like hybrid kinases are not restricted to Gram-positive bacteria, but also occur in Proteobacteria, Cyanobacteria and Bacteroidetes. However, it seems that in these phyla the RsbK-type HKs are used for purposes other than the regulation of alternative sigma factors. This finding is similar to what has been observed for the RsbRST-like modules which occur in a wide variety of bacteria, where they appear to interact with different types of downstream signalling modules (Pané-Farré *et al.*, 2005). Most of these downstream modules were found to include hybrid HKs (unrelated to RsbK) with up to two REC domains, indicating complex phophorelays. Interestingly, a universal feature of these downstream modules was the presence of a PP2C-type phosphatase, related to RsbX (Fig. 1a). These findings indicated that the stressosome together with its cognate PP2C-type phosphatase protein function as a 'core' module for gathering signals and conveying these to diverse downstream signalling modules (Pané-Farré *et al.*, 2005). As shown in this study, RsbK-type HKs are also often connected to potential complex downstream signalling modules, including different types of RRs with additional C-terminal signalling domains. Apparently, RsbRST- and RsbK-like modules provide a common solution to the problem of signal integration in bacteria. However, despite the apparently universal use of these signalling modules across bacteria, considerable variability may arise within these modules. In the case of the RsbK-type HKs, the variability even occurs between homologues that are potentially involved in the same process, as is demonstrated by the difference in N-terminal sensory domains between RsbK and OsaA. The observed variation reflects the different niches in which the associated organisms reside and may illustrate the common yet specific solutions these organisms have evolved for the same process: the control of a stress-responsive alternative sigma factor. Finally, our findings further demonstrate the highly modular nature of sigma factor activation routes and signal transduction routes in general.

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